MATERIALS AND METHODS FOR MOLECULAR DETECTION OF CLINICALLY RELEVANT, PATHOGENIC FUNGAL SPECIES

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FIELD OF THE INVENTION

This invention relates to the field of molecular biology and to the detection of detrimental fungal species. More specifically, the invention provides materials and methods which facilitate the identification of pathogenic fungal species in infected patients.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals in parentheses in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are set forth at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

Fungi are eukaryotic microorganisms that are universally distributed. In nature, fungi play a major role in the decomposition of plant materials. They are also responsible for spoilage of food and in the preparation of beverages and pharmaceuticals. Out of an estimated 100,000 species of fungi described by mycologists, approximately 150 species are recognized as pathogens in humans and animals. The increasing incidence of AIDS and the development of newer treatments for hematologic malignancies and solid organ transplants has led to an increase in the number of immunocompromised patients. These patients have a high risk of developing fungal infections, which, if not rapidly diagnosed and treated, are capable of causing death in a matter of days. The number of antifungal drugs is limited and their toxic side

effects on the patient are much higher than that of comparable antibacterial therapy. Therefore, a rapid diagnosis of a fungal infection with administration of appropriate therapy is critical in these patients.

Aspergillus species, for example, are associated with allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis, and invasive infection. The most severe disease caused by the aspergilli occurs in immunocompromised patients with invasive pulmonary infection followed by rapid dissemination. The frequency of invasive aspergillosis (IA), as well as other invasive mold infections has increased in recent years due to the expanding number of patients receiving aggressive chemotherapy regimens and immunosuppressive agents (2). The nonspecific symptoms and the lack of rapid diagnostic assays to detect these infections have been major problems in treating patients with invasive disease Early recognition of invasive fungal infection and treatment with appropriate antifungal therapy is key to reducing the mortality associated with disseminated disease (25). The mortality rate for bone marrow transplant patients with pulmonary IA is greater than 70% (5, 15). Due to the typically long time required for identification of a mold using standard culture procedures, most patients with suspected disease are treated empirically with amphotericin B (AmB). Resistance to AmB as well as itraconazole has been reported for some *Aspergillus* species although the number of isolates studied in each case is limited (14, 16).

Unfortunately, the identification of the aspergilli based on morphologic methods requires adequate growth for evaluation of colony characteristics and microscopic features. A culture time of 5 days or more is generally required for identification of anamorphic forms of *Aspergillus*. There are

more than 180 species in the *Aspergillus* genus, although three, including *A. flavus*, *A. fumigatus* and *A. terreus* account for the vast majority of IA infections. *A. nidulans*, *A. niger* and *A. ustus* are rarely encountered as causes of invasive disease(18).

Various molecular approaches have been used for the detection of *Aspergillus* from environmental and clinical samples (3, 6, 27). Targets for the genus-level detection of *Aspergillus* have included the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region, and the internal transcribed spacer (ITS) regions. The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome. The rRNA gene for 5.8S RNA separates the two ITS regions. The sequence variation of ITS regions has led to their use in phylogenetic studies of many different organisms (9, 26).

It would be highly advantageous if means were available to differentiate and efficiently identify clinically relevant pathogenic fungi. It is an object of the present invention to provide compositions, methods and kits to accomplish this goal.

SUMMARY OF THE INVENTION

Numerous fungi, once thought to be nonpathogens, have emerged as causes of human disease (31,32,34). The identification of these unusual fungal pathogens is difficult, frequently requiring the expertise of a fungal reference laboratory. The uniqueness of the nucleic acid sequence of the ITS regions allows for the identification of these novel pathogens (30,31,32,34).

In accordance with the present invention, materials and methods are provided which facilitate the differentiation and identification of clinically relevant pathogenic fungal species. In one aspect of the invention, a universal primer set having SEQ ID NOS: 1 and 2 suitable for amplifying ITS amplicons from a wide variety of pathogenic fungal species is provided.

In a preferred embodiment of the invention, methods are disclosed for identifying pathogenic fungal species by virtue of species specific differences in the ITS regions amplified using the universal primer set of the invention. The method comprises determining whether one or more fungal species selected from the group of fungal species consisting of *Aspergillus ustus, Aspergillus terreus, Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Pseudallescheria boydii, Fusarium solani, Fusarium oxysporum, Fusarium monilliformes, Penicillium spp., Malassezia furfur, Malbarnchia spp., Cylindrocarpon lichenicola, Cladophialophora bantiana, Arthrogrothilus spp., Gymnascella hyalinaspora, Cylindrocarpon destructans, Sporothrix schenkii, Blastomyces dermatitides, Penicillium marnefeii, Histoplasma duboisii, Histoplasma capsulatum, Coccidiodes immitis, Cryptococcus neoformans, Issatchenkia orientalis, Candida albicans, Candida tropicalis, Candida lusitaniae, Candida glabrata, and Candida parapsilosis*, is present in a biological sample. The steps of the method include a) extracting nucleic acid material from fungi contained in the sample; b) adding two known oligonucleotide primers, one of the primers being (SEQ ID NO:1) and the other primer being (SEQ ID NO:2), the primers bracketing a hypervariable region on the rRNA present in the fungal species of the group; c) amplifying the sequence between the primers; and d) using one or more detectably

labeled probes directed to a portion of the hypervariable region bracketed by the primers, each of the labeled probes being specific for one of the fungal species from said group. In a preferred embodiment the target ITS sequences are amplified by polymerase chain reaction. The one or more probes recited in the method are selected from the group consisting of (SEQ ID NO:3), (SEQ ID NO:4), (SEQ ID NO:5), (SEQ ID NO:6), (SEQ ID NO:7), (SEQ ID NO:8), (SEQ ID NO:9), (SEQ ID NO:10), (SEQ ID NO:11), (SEQ ID NO:12), (SEQ ID NO:13), (SEQ ID NO:14), (SEQ ID NO:15), (SEQ ID NO:16), (SEQ ID NO:17), (SEQ ID NO:18), (SEQ ID NO:19), (SEQ ID NO:20), (SEQ ID NO:21), (SEQ ID NO:22) and (SEQ ID NO:23), (SEQ ID NO: 24),(SEQ ID NO:25),(SEQ ID NO:26),(SEQ ID NO:27),(SEQ ID NO:28), (SEQ ID NO:29),(SEQ ID NO:30), and (SEQ ID NO:31), (SEQ ID NO:32), (SEQ ID NO:33), each having a characteristic ITS sequence indicating fungal species of origin. In a further embodiment of the method, a plurality of probes are used in step (d each being connected to (a) a different signal moiety or (b) a moiety which allows separation of said probes.

The following fungal specific oligonucleotide sequences for use as probes in the methods of the invention are also provided herein. An oligonucleotide sequence specific for *Penicillium spp.*, having the nucleotide sequence of (SEQ ID NO:25) or the complement thereof; an oligonucleotide sequence specific for *Malbranchia spp.*, having the sequence of (SEQ ID NO:26) or the complement thereof; an oligonucleotide sequence specific for *Arthorgrothilus spp.*, having the sequence of (SEQ ID NO:27) or the complement thereof; an oligonucleotide sequence specific for *Cylindrocarpon destructans*, having the sequence of (SEQ ID NO:28) or the complement thereof; an oligonucleotide sequence specific for *Sporothrix schenkii*, having the sequence of (SEQ ID NO:29) or the complement

thereof; an oligonucleotide sequence specific for *Penicillium marnefeii*, having the sequence of (SEQ ID NO:30) or the complement thereof; an oligonucleotide sequence specific for *Coccidiodes immitis*, having the sequence of (SEQ ID NO:31) or the complement thereof; an oligonucleotide sequence specific for *Candida tropicalis*, having the sequence (SEQ ID NO:32) or the complement thereof; an oligonucleotide sequence specific for *Candida parapsilosis*, having the sequence of (SEQ ID NO:33) or the complement thereof.

Also within the scope of the present invention, are kits for identifying pathogenic fungal species in a biological sample. The kits comprise 1) a universal primer set, having the sequence of SEQ ID NO: 1 and SEQ ID NO: 2; 2) lysis buffer suitable for lysing fungus in the biological sample, such that DNA is released from the fungus upon exposure to said buffer; 3) a polymerase enzyme suitable for use in PCR 4) means for contacting said released DNA with a primer set having the sequence of SEQ ID NO: 1 and NO: 2 under conditions where amplification of pathogenicity-associated ITS sequences occurs, if said pathogenic fungus is present in said sample; and 5) means for detecting said amplified sequence, if present. In a further embodiment, the kit of the invention, contains sequences having SEQ ID NOS: 3-33 for comparing the amplified fungal ITS sequence thereby identifying said pathogenic fungus if present.

With reference to nucleic acids used in the invention, the term "isolated nucleic acid" is sometimes employed. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may

comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryotic or eucaryotic cell. An "isolated nucleic acid molecule" may also comprise a cDNA molecule. An isolated nucleic acid molecule inserted into a vector is also sometimes referred to herein as a "recombinant" nucleic acid molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

With respect to single stranded nucleic acids, particularly oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the

substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. Appropriate conditions enabling specific hybridization of single stranded nucleic acid molecules of varying complementarity are well known in the art.

For instance, one common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is set forth below (Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (29):

T_m = 81.5°C + 16.6Log [Na+] + 0.41(% G+C) - 0.63 (% formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57° C. The T_m of a DNA duplex decreases by 1 - 1.5° C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42 °C.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be

either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The phrase "solid matrix" as used herein includes, without limitation, filter paper, multiwell dishes, microchips, derivatized magnetic particles and the like.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable

cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described in US Patents 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the nucleotide sequence alignment *of A. flavus* (ATCC 16883), *A. fumigatus* (ATCC 36607), *A. nidulans* (ATCC 10074), *A. niger* (ATCC 16888), *A. terreus* (ATCC 16792),

and *A. ustus* (ATCC 301953). The alignment consists of the 3' end of the 18S ribosomal RNA (rRNA) gene , the complete ITS region, the complete ITS 2 region, and the 5' end of the 28S rRNA gene. The highly conserved 5.8S rRNA gene sequence has been omitted.

DETAILED DESCRIPTION OF THE INVENTION

Each of the cells of all life forms, except viruses, contain ribosomes and therefore the gene for ribosomal RNA. A ribosome contains three separate single-stranded RNA molecules, namely, a large molecule, a medium sized molecule, and a small molecule. The two larger rRNA molecules vary in size in different organisms.

Ribosomal RNA is a direct gene product and is coded for by the rRNA gene. This DNA sequence is used as a template to synthesize rRNA molecules. A separate gene exists for each of the ribosomal RNA subunits. Multiple rRNA genes exist in most organisms and many higher organisms contain both nuclear and mitochondrial rRNA genes. Plants and certain other organisms contain nuclear, mitochondrial and chloroplast rRNA genes. The rRNA gene and gene product have been well characterized in certain species. Hybridization of rRNA and ribosomal genes in genetic analysis and evolution and taxonomic classification of organisms and ribosomal gene sequences has been described. Genetic analysis may include, for example, the determination of the numbers of rRNA genes in various organisms; the determination of the similarity between the multiple rRNA genes which are present in cells; determination of the rate and extent of synthesis of rRNA in cells and the factors which control them.

In accordance with the present invention, specific sequences have been selected which allow for the

identification of clinically relevant pathogenic fungal species unambiguously. The selection of specific sequences is based on differences in the internal transcribed spacer molecules between rRNA genes. These sequences may be used to advantage in methods routinely practiced in the laboratory setting.

The present invention is directed to compositions and methods utilizing sequences from phylogenetically informative segments of rRNA genes from a large variety of fungal isolates.

I. PREPARATION OF NUCLEIC ACID MOLECULES AND PRIMERS WHICH DIFFERENTIATE AMONG FUNGAL SPECIES

A. NUCLEIC ACID MOLECULES

Extraction of DNA from fungi was performed following the needle inoculation of 50 ml of Sabouraud dextrose (SAB) broth (Difco Laboratories: Detroit, MI) with conidia from a 7 day culture from SAB agar and incubation for 72 h at 30 °C. The hyphae were recovered on a 0.45 μm filter and washed with sterile saline. Aliquots of the fungal hyphae were stored frozen at -70 °C until use. Prior to lysis, the hyphae were thawed and suspended in 400 μl of DNA extraction buffer (1mM EDTA, pH 8.0; 1% sodium dodecyl sulfate, 10 mM Tris-HCL, pH 7.6; 100 mM NaCl, 2% Triton X100) as described by Van Burik *et al.* (24). Microcentrifuge tubes (1.5 ml.) containing hyphae and buffer were sonicated in a water bath (Branson, Model 2210) for 15 m followed by heating at 100 °C for 5 m. Following lysis, DNA was purified using the QIAmp blood kit (Qiagen Inc, Valencia, CA) and protocols for crude cell lysates as supplied by the manufacturer. Following extraction, the purified DNA was stored at 4 °C until tested. Extraction of DNA from blood, paraffinized tissue or other

clinical material is performed by adding a small sample of specimen to the fungal DNA extraction buffer and following the procedure as outlined above.

PRIMER PREPARATION AND SEQUENCE COMPOSITION

Nucleic acid molecules encoding the differentiating oligonucleotides of the invention may be prepared by synthesis from appropriate nucleotide triphosphates, a method utilizing protocols well known in the art. The availability of nucleotide sequence information, such as primers having the sequence of SEQ ID NO:1 or SEQ ID NO:2 enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant primers may be used according to methods known in the art, such as he polymerase chair reation (PCR) method.

Informative ITS sequences from 31 different fungal isolates are provided herein as SEQ ID NOS: 3-33. See Addendum 1. Accordingly, specific probes may be developed for identifying the specific fungi.

In accordance with the present invention, nucleic acids primers having the appropriate level of sequence homology with the sequences provided herein may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al.,(1989, *supra*), using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42° C for at

least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37° C in 1X SSC and 1% SDS; (4) 2 hours at 42-65 °C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

The nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the sequences provided herein. Also contemplated in the scope of the present invention are oligonucleotide probes which specifically hybridize with the DNA from pathogenic species of fungus under high stringency conditions. Primers capable of specifically amplifying the ITS segments of fungal rDNA encoding nucleic acids described herein are also contemplated to be within the scope of the present invention. As mentioned previously, such oligonucleotides are useful as primers for detecting, isolating and amplifying sequences associated with pathogenic fungus. SEQ ID NOS: 1 and 2 are a suitable universal primer set for this purpose.

It will be appreciated by persons skilled in the art that variants (e.g., allelic variants) of the ITS sequences exist in the fungus population, and must be taken into account when designing and/or utilizing oligonucleotides of the invention. Accordingly, it is within the scope of the present invention to encompass such variants, with respect to the ITS sequences disclosed herein or the oligonucleotides targeted to specific locations on the respective genes or RNA transcripts. Accordingly, the term "natural allelic variants" is used

herein to refer to various specific nucleotide sequences of the invention and variants thereof that would occur in a population. The occurrence of genetic polymorphisms which give rise to minor base changes in a DNA molecule are known to those of ordinary skill in the art. Additionally, the term "substantially complementary" refers to oligonucleotide sequences that may not be perfectly matched to a target sequence, but such mismatches do not materially affect the ability of the oligonucleotide to hybridize with its target sequence under the conditions described.

IDENTIFICATION OF FUNGAL SPECIES BY SEQUENCE ANALYSIS AND/OR PROBE HYBRIDIZATION.

Currently, the most direct method for the identification of fungi is DNA sequence analysis however the methodology is also labor intensive and expensive. It is usually not practical to sequence all potentially relevant regions of every experimental sample. Other exemplary approaches for recognizing species of fungi based on nucleic acid differences include:

a) comparing the sequence of nucleic acid in the sample with nucleic acid sequences from the non-pathogenic and pathogenic species of fungus to determine which species is responsible for infection in the patient; or

b) using DNA restriction mapping to compare the restriction pattern produced when a restriction enzyme cuts a sample of nucleic acid from the sample as compared with the restriction pattern obtained from pathogenic and non-pathogenic species of fungus, or,

c) using a specific binding member capable of binding to a either the pathogenic nucleic acid sequence, the

specific binding member comprising nucleic acids which distinguish between fungal species based on hybridization specificities, or substances comprising an antibody domain with specificity for a pathogenic or non-pathogenic fungal nucleic acid sequence, the specific binding member being labeled so that binding of the specific binding member to its binding partner is detectable; or

d) in situ hybridization between fungal DNA from permeabilized tissue sections and fluorescent molecular probes specific for pathogenic fungal species under investigation; or

e) using PCR involving one or more primers based pathogenic fungal gene sequences to screen for the presence of the pathogenic species in a sample.

A "specific binding pair" comprises a specific binding member (sbm) and a binding partner (bp) which have a particular specificity for each other and which under normal conditions bind to each other in preference to other molecules. Examples of specific binding pairs are antigens and antibodies, ligands and receptors and complementary nucleotide sequences. The skilled person is aware of many other examples and they do not need to be listed here. Further, the term "specific binding pair" is also applicable where either or both of the specific binding member and the binding partner comprise a part of a large molecule. In embodiments in which the specific binding pair are nucleic acid sequences, they will be of a length to hybridize to each other under conditions of the assay, preferably greater than 10 nucleotides long, more preferably greater than 15 or 20 nucleotides long.

In most embodiments for screening for the presence of pathogenic fungus the nucleic acid associated with the pathogenic phenotype in the sample will initially be

amplified, e.g. using PCR, to increase the amount of the analyte as compared to other sequences present in the sample. This facilitates target sequences detection with a high degree of sensitivity if such sequences are present in the sample. This initial step may be avoided by using highly sensitive array techniques that are becoming increasingly important in the art.

The identification of the specific nucleic acid associated with fungal pathogenicity paves the way for aspects of the present invention to provide the use of materials and methods, such as are disclosed and discussed above, for rapid detection of the presence or absence in a test sample of the fungal pathogen and to identify the fungus to species.

The invention allows for planning of appropriate quarantine and/or prophylactic measures and permits rapid determination of diagnosis and treatment of infected patients.

The following examples are provided to illustrate embodiments of the invention. They are not intended to limit the invention in any way.

EXAMPLE I

IDENTIFICATION OF *ASPERGILLUS* SPECIES USING INTERNAL TRANSCRIBED SPACER REGIONS 1 AND 2

The following protocols are provided to facilitate the practice of the present invention (taken from a modification of Henry et al.[30]).

Cultures for analysis.

Referenced cultures of *Aspergillus* species obtained from the American Type Culture Collection (ATCC) included *Aspergillus flavus* ATCC 16883, *A. fumigatus* ATCC 36607, *A.*

nidulans ATCC 10074, *A. niger* ATCC 16888, and *A. terreus* ATCC 16792. *A. ustus* was obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH 9479). Isolates of *Aspergillus* species from cases of IA were obtained from patient samples catalogued at the University of Nebraska Medical Center (UNMC) and inventoried in the Invasive Molds Infection database. Morphologic identification of clinical isolates to the species level was accomplished using established procedures including microscopic and macroscopic characteristics. Additional fungal species selected for sequence comparison with *Aspergillus* reference strains are *Ajellomyces capsulatus, Ajellomyces dermatitidis, Candida albicans, Cladophialophora bantiana, Cryptococcus neoformans, Cylindrocarpon lichenicola, Fusarium oxysporum, Fusarium solanii, Fusarium spp., Gymnascella hyalinospora, Penicillium capsulatum, Penicillium glabrum, Penicillium marnefeii, Phialophora verrucosa, Pseudallescheria boydii, and Saccharomyces cerevisiae.*

Culture preparation and DNA extraction.

Extraction of DNA from fungi was performed following the needle inoculation of 50 ml of Sabouraud dextrose (SAB) broth (Difco Laboratories: Detroit, MI) with conidia from a 7 day culture from SAB agar and incubation for 72 h at 30 °C. The hyphae were recovered on a 0.45 μm filter and washed with sterile saline. Aliquots of the fungal hyphae were stored frozen at -70 °C until use. Prior to lysis, the hyphae were thawed and suspended in 400 μl of DNA extraction buffer (1mM EDTA, pH 8.0; 1% sodium dodecyl sulfate, 10 mM Tris-HCL, pH 7.6; 100 mM NaCl, 2% Triton X100) as described by Van Burik *et al.* (24). Microcentrifuge tubes (1.5 ml.) containing hyphae and buffer were sonicated in a water bath (Branson, Model

2210) for 15 m followed by heating at 100 °C for 5 m. Following lysis, DNA was purified using the QIAmp blood kit (Qiagen Inc, Valencia, CA) and protocols for crude cell lysates as supplied by the manufacturer. Following extraction, the purified DNA was stored at 4 °C until tested.

Primers.

Modifications of the original primers as stated by Henry et al were made to optimize the amplification procedure (30). The modified 5' primer is 5'GGA AGT AAA AGT CGT AAC AAG G 3' (SEQ ID NO: 1) and the modified 3' primer is 5' GTA TCC CTA CCT GAT CCG AGG 3' (SEQ ID NO: 2). These primers make use of conserved regions of the 18S (SEQ ID NO: 1) and the 28S (SEQ ID NO:2) rRNA genes to amplify the intervening 5.8S gene and the ITS 1 and ITS 2 non-coding regions. Primers were synthesized by the UNMC /Eppley Molecular Biology Core Laboratory.

PCR amplification.

The PCR assay was performed with 5 μl of test sample in a total reaction volume of 50 μl consisting of PCR Buffer (20 mM Tris-HCl (pH 8.4) and 50 mM KCl); 0.1 mM (each) dATP, dGTP, dCTP, and dTTP; 1.5 mM $MgCl_2$; 0.3 μM (each) primer; and 1.5 U of platinum *Taq* DNA polymerase high fidelity (GibcoBRL, Life Technologies, Gaithersburg, MD). Forty cycles of amplification were performed in a Stratagene Robocycler Model 96 thermocycler after initial denaturation of DNA at 95 °C for 4.5 m. Each cycle consisted of a denaturation step at 95 °C for 30 s, and annealing step at 50 °C for 30 s, and extension step at 72 °C for 1 m, with a final extension at 72 °C for 3 m

following the last cycle. After amplification, the products were stored at 4 °C until used.

Cloning of PCR products.

Amplicons were separated by agarose gel electrophoresis, purified and ligated into the pCR 2.1 plasmid vector using the Invitrogen Original TA Cloning Kit (Invitrogen, San Diego, CA). Competent INV F' One Shot cells were transformed using standard protocols. Colonies were isolated and purified with a Qiagen mini-prep spin kit according to the manufacturer's protocols. An aliquot of purified plasmid was digested with EcoRI endonuclease (New England Biolabs, Beverly, MA) and screened by agarose gel electrophoresis for the presence of a 300-bp doublet corresponding to the presence of an EcoRI cleavage site, GAATTC, within the 5.8S sequence. Selected plasmids were submitted to the Eppley Molecular Biology Core Laboratory for automated dye termination sequencing.

DNA sequencing.

DNA sequencing was performed at the Eppley Molecular Biology Core Laboratory on a Perkin Elmer/ABI Model 373 DNA sequencer with protocols supplied by the manufacturer. For sequencing of cloned fragments, both strands of the plasmid containing fungal insert were sequenced with universal M13 forward and reverse sequencing primers. For direct sequencing of non-cloned amplicons, PCR products were directly sequenced using the SEQ ID NO: 1 and the SEQ ID NO: 2 PCR primers. The resultant nucleotide sequences were aligned with the MacVector sequence analysis software Version 6.5 (Oxford Molecular Group, Inc., Campbell, CA) alignment application.

GenBank accession numbers.

The ITS 1 - 5.8S - ITS 2 rRNA gene complex sequences of referenced *Aspergillus* species not previously available within the National Center for Biotechnology Information (NCBI) GenBank or European Molecular Biology Laboratory (EMBL) databases were submitted to GenBank. The assigned sequence accession numbers are *A. flavus* (ATCC 16883) as AF138287 (SEQ ID NO:8), *A. fumigatus* (ATCC 36607) as AF138288 (SEQ ID NO: 7), *A. niger* (ATCC 16888) AF138904, *A. terreus* (ATCC 16792) AF138290, and *A. ustus* (ATCC 201953) AF157507. *A. nidulans* (ATCC 10074) AF138289 was accepted into GenBank as *Emericella nidulans*. Sequences from other fungal species also deposited into GenBank are *Ajellomyces capsulatus* AF038353, *Candida albicans* AF217609, *Cladophialophora bantiana* AF131079, *Cryptococcus neoformans* AF162916, *Cylindrocarpon lichenicola* AF133845, *Gymnascella hyalinospora* AF129854, and *Pseudallescheria boydii* AF181558.

Sequence analysis.

Sequence comparisons of referenced strains and clinical isolates were made using MacVector 6.5 software (Oxford Molecular Group, Inc.) and the Clustal W alignment algorithm. Intra-species sequence similarity and variation for isolates was determined by the MacVector software and visually confirmed using pairwise nucleotide alignments. Sequences from referenced isolates were aligned to complete or partial ITS sequences available in GenBank after submission of sequence data from this study. Comparison of sequences from referenced isolates, clinical isolates and GenBank sequences was performed using a non-gapped, advanced BLAST search (1). The similarities of the sequences were determined with the expectation frequency minimized to 0.0001. Sequences were not

filtered for low complexity.

Clinical isolate identification study.

Eleven isolates of various *Aspergillus* species previously identified by the UNMC Mycology Laboratory were selected by one of us (PI) and innoculated onto Sabouraud dextrose agar and incubated at 30 °C for 24 hours. Isolates included 3 *A. fumigatus*, 2 *A. flavus*, *1 A. ustus*, 2 *A. terreus*, 2 *A. niger*, and 1 *A. nidulans*. The plates were coded and the presented for processing by a second person (TH). An approximate 2 mm^3 section of the agar at the site of innoculation was taken for DNA extraction and amplification. The amplicons were purified using the Qiagen PCR Purification Kit (Qiagen Inc.) and sequenced directly. Sequence analysis of *Aspergillus* specimens was performed using an advanced, non-gapped BLAST search with expectation frequency set to 0.0001 and no filtering for low complexity. The search was performed following the deposition and acceptance of sequences from referenced isolates into GenBank. Species identification was determined from the highest bit score of the species listed from the BLAST search. The amount of time from submission of the culture plates to identification was determined.

RESULTS

Analysis of the ITS regions.

Amplification of the ITS 1 - 5.8S - ITS 2 regions from the six clinically relevant *Aspergillus* strains generated PCR products ranging in size from 555 to 603 bp (**Table 1**).

TABLE 1.

Aspergillus species PCR products.

Aspergillus species	Source	Size (bp)[a]
A. flavus	ATCC 16883	585
A. flavus	clinical isolate	585
A. fumigatus	ATCC 36607	586
A. fumigatus	clinical isolate	588
A. nidulans	ATCC 10074	555
A. nidulans	clinical isolate	559
A. niger	ATCC 16888	589
A. niger	clinical isolate	589
A. terreus	ATCC 16792	599
A. terreus	clinical isolate	603
A. ustus	UAMH 9479	560
A. ustus	clinical isolate[b]	560

Abbreviations: ATCC, American Type Culture Collection; UAMH, University of Alberta Microfungus Collection and Herbarium. [a]Includes the complete ITS 1, 5.8S, ITS 2 regions and portions of the 18S (54 bp) and 28S (25 bp) rRNA genes. [b]Deposited into the American Type Culture Collection as ATCC 201953.

Sequencing was first performed on cloned amplicons and then repeated using direct sequencing of PCR products with comparisons made between results from both methods. Although a *Taq* polymerase with proofreading capability was used in generation of amplicons, an examination was made for potential variation in sequence due to random base changes introduced by the amplification process. Two clones from each reference strain for each species were sequenced. The sequence of cloned PCR products varied no more than 2 nucleotides from the sequence of amplicons directly sequenced. Minimal differences in amplicon length were seen between referenced and clinical strains of the same species.

Alignment of contiguous fungal sequences demonstrated that both single nucleotide differences and short lengths of sequence diversity due to insertions or deletions existed in the ITS 1- 5.8S - ITS 2 regions among the pathogenic *Aspergillus* species (Figure 1). The ITS 1 region displayed

more inter-species variation than the ITS 2 region, with approximately four separate variable regions. ITS 2 contained two variable regions ranging from 6 to 10 bp in length. A matrix analysis of the sequence similarity between ITS 1 and 2 sequences of the referenced *Aspergillus* species is depicted in **Table 2**. The greatest similarity among pathogenic species existed between *A. fumigatus* and *A. niger* with 52 nucleotide base differences (91.7% similarity) whereas *A. ustus* showed the greatest diversity when compared with *A. terreus*, with differences at 128 nucleotide positions (79.3% similarity).

TABLE 2.

Matrix of ITS 1-5.8S-ITS 2 similarities for referenced Aspergillus species.

	A. flavus ATCC 16883	*A.fumigatus* ATCC 36607	*A. nidulans* ATCC 10074	*A. niger* ATCC 1688	*A. terreus* ATCC 16792	*A. ustus* ATCC201953
A. flavus ATCC 16883						
A. fumigatus ATCC 36607	87.6					
A. nidulans ATCC 10074	81.5	84.3				
A. niger ATCC 16888	89.6	91.7	84.0			
A. terreus ATCC 16792	87.0	91.1	83.0	90.6		
A. ustus ATCC 201953	82.7	80.7	91.4	80.5	79.3	

Aspergillus ITS sequences generated in our laboratory from ATCC strains were compared with all *Aspergillus* sequences available in GenBank following the deposition of sequences listed in **Table 3**. For *A. flavus*, *A. fumigatus*, or *A. terreus*, the inter-species sequence similarity with all *Aspergillus*

GenBank sequences (referenced and non-referenced) was found to be less than 99%. Sequence similarity of 99% was observed between *A. nidulans* (accepted into GenBank as *Emericella nidulans)* and *Emericella quadrilineata*. Sequence similarity of 99% was also found among species within the *A. niger* aggregate including *A. phoenicis* and *A. tubigensis*.

09580797.053000

TABLE 3.
Number of nucleotide differences in ITS 1, 5.8S and ITS 2 within a single species.

Species and accession number	No. of nucleotide base differences ITS1	ITS 2	ITS1-5.8S- ITS 2	% similarity
Aspergillus flavus ATCC 16883				
IMI 210	0	1	1	99.8
AB008414	0	1	1	99.8
AB008415	0	2	2	99.7
AB008416	0	0	0	100.0
AF027863	0	0	0	100.0
AF078893	0	1	1	99.8
AF078894	0	0	0	100.0
L76747	4	0	4	99.3
Aspergillus fumigatus ATCC 36607				
IMI 196	2	2	5	99.2
AF078889	2	0	2	99.7
AF078890	1	0	1	99.8
AF078891	1	0	1	99.8
AF078892	1	0	1	99.8
Aspergillus nidulans ATCC 10074				
IMI 231	2	2	4	99.3
L76746	0	0	0	100.0
U03521	NA	2	2	99.6
Aspergillus niger ATCC 16888				
IMI 026	0	0	0	100.0
AF078895	0	0	0	100.0
AJ223852	4	1	5	99.2
L76748	0	0	0	100.0
U65306	0	0	0	100.0
Aspergillus terreus ATCC 16792				
IMI 203	4	0	4	99.3
AF078896	0	0	0	100.0
AF078897	0	0	0	100.0
AJ001334	0	0	0	100.0
AJ001335	0	0	0	100.0
AJ001338	0	0	0	100.0
AJ001368	0	1	1	99.8
L76774	0	0	0	100.0
U93684	0	3	3	99.5
Aspergillus ustus UAMH 9479				
IMI 192b	0	1	1	99.8

Abbreviations: ATCC, American Type Culture Collection; IMI, Invasive Moulds Infections (UNMC); UAMH, University of Alberta Microfungus Collection and Herbarium. aDeposited into GenBank as Emericella nidulans. bDeposited into the American Type Culture Collection as ATCC 201953.

Sequence similarity of clinical isolates and reference strains of the same species.

The results of comparisons between clinical isolates and referenced strain sequences of the same *Aspergillus* species are shown in Table 3. The greatest intra-species variation was seen among isolates of *A. fumigatus* and isolates of *A. niger*. For both species, 5 nucleotide base differences existed between the sequence of clinical isolates and the referenced strain. Considering the length of the ITS region amplified, the overall sequence similarity was greater than 99% between the referenced *Aspergillus* strains and clinical isolates of the same species.

Sequence comparisons with other true pathogenic and opportunistic fungi.

To evaluate the utility of ITS sequences for identification of true pathogenic and opportunistic fungi, the ITS 1, 5.8S, and ITS 2 region sequences of 12 different genera known to cause infection in humans were determined in our laboratory and compared to sequences from the six medically important aspergilli. The results obtained with *A. fumigatus* are shown in Table 4. In comparison with *A. fumigatus*, sequence similarities among listed genera ranged from 50.2% to 89.6 %, with *Penicillium* species showing the greatest sequence similarity. BLAST search comparisons were also made between the other medically important Aspergillus species and all opportunistic fungi available in the GenBank database (data not shown). The ITS 1 and 2 sequences of the referenced *Aspergillus* species differed from the other fungal genera by at least 1%, with one exception; *A. niger* ITS sequences had 99% sequence similarity with *Arthrobotrys* species and *Gliocladium cibotii*. As expected, the referenced *A. niger* sequence was listed first in the bit score rank listing. To further test

the system, the sequences of clinical isolates of *A. niger* were compared using an ungapped BLAST search of the GenBank database. In each case, the clinical isolate was distinguished from *Arthrobotrys* species, and *Gliocladium cibotii* on the basis of bit score.

TABLE 4.
Nucleotide base differences of ITS 1-5.8S-ITS 2 between A. fumigatus and other medically important fungal genera.

Species and accession number	No. of nucleotide base differences ITS 1	ITS 2	ITS 1-5.8S-ITS 2	% similaritya
Ajellomyces capsulatus				
AF038353	93	45	143	76.6
AF156892b	86	59	150	76.7
Ajellomyces dermatitidis				
AF038355	93	66	163	74.1
Candida albicans				
AF217609b	108	98	221	65.0
L28817	97	98	211	64.8
Cladophialophora bantiana				
AF131079b	82	111	202	68.1
Cryptococcus neoformans				
AF162916b	99	123	237	59.1
L14067	53	126	193	59.4
Cylindrocarpon lichenicola				
AF133845b	102	79	185	69.2
Fusarium oxysporum				
AF132799	85	91	180	62.3
Fusarium solanii				
U38558	100	92	202	66.7
Fusarium spp.				
IMI 183	99	89	197	67.0
Gymnascella hyalinospora				
AF129854b	87	57	149	76.3
Penicillium capsulatum				
AF033429	44	23	70	88.0
Penicillium glabrum				
AF033407	39	22	62	89.6
Penicillium marnefeii				
ATCC 18224c	60	57	124	79.1
L37406	57	54	116	79.5
Phialophora verrucosa				
AF050281	78	104	196	67.5
Pseudallescheria boydii				
AF022486	106	131	248	55.6
AF181558b	109	132	252	55.4
Saccharomyces cerevisiae				
Z95929	144	146	302	50.2

Abbreviations: ATCC, American Type Culture Collection; IMI, Invasive Mold Infections (UNMC); aAs compared to A. fumigatus ATCC 36607. bSequence deposited into GenBank as part of this study. cReference strain sequenced but not deposited into GenBank.

Clinical validation of ITS sequence analysis.

To determine the utility of the ITS sequence for accurate identification of *Aspergillus* species, a blinded comparison was made using 11 morphologically confirmed *Aspergillus* clinical isolates. Following incubation of the culture plate for 24 hours at 30° C, and direct sequencing of PCR amplicons, ITS sequences were used in an ungapped BLAST search of the GenBank database. Identification of the unknown sequences was made using the highest bit score of listed species. Using this method, each of the coded specimens was identified correctly as the appropriate *Aspergillus* species. All of the identifications were made in less than 48 hours after receipt of the blinded culture plate.

DISCUSSION

The increasing frequency of invasive fungal infection and the high mortality associated with disseminated fungal disease has highlighted the need for rapid identification of infectious molds from clinical samples. The number of cases of invasive aspergillosis (IA) found at autopsy has increased 14-fold since 1978 (8). Early recognition and treatment of patients with invasive fungal infection is crucial, as the progression of invasive disease from detection to death is typically less than 14 days (4, 25). The present work was based on the premise that identification of *Aspergillus* at the species level will have clinical importance in the future. Currently, physicians rely on clinical findings and administer amphotericin B (AmB) empirically to immunosuppressed patients

with sign and symptoms consistent with a fungal infection. However, the resistance of certain *Aspergillus* species to antifungal agents complicates empiric treatment for invasive disease (4, 14, 16). The effectiveness of AmB varies significantly depending on the species of *Aspergillus*, with over 95% of *A. terreus* isolates reported as resistant (10, 17, 22). Susceptibility testing has revealed a wide range of AmB MIC values; from 0.5 μg/ml for *A. niger* and *A. fumigatus* to 16 μg/ml for *A. flavus* and *A. nidulans*. Thus, rapid diagnosis and recognition of the species causing infection and treatment with the most active antifungal therapy may be important to reducing the mortality of immunosuppressed patients with IA.

The detection of *Aspergillus* DNA has been accomplished from blood, serum, bronchoalveolar lavage fluid, and tissue using the 18S rRNA gene as the target (6, 12, 24, 28). Einsele *et al.* detected *Aspergillus* DNA from blood approximately four days prior to the appearance of pulmonary infiltrates consistent with fungi by CT scan in patients with presumed aspergillosis (6). While their report detailed the shortened time-span to positive identification of *Aspergillus* from patient material, it was not possible to identify *Aspergillus* at the species level using the18S rRNA gene (12). Additionally, the identification of aspergilli by PCR in some patient specimens, such as bronchoalveolar lavage fluid, does not always indicate invasive disease and therefore the use of PCR for detection of fungi in specimens from potentially colonized sites may be limited.

The ITS regions have been used as targets for phylogenetic analysis because they generally display sequence variation between species, but only minor variation within strains of the same species (11, 13, 20, 21). Shin *et al.* have described a fluorescent DNA probe assay using the ITS 2 region

for the identification of *Candida* species (19). Their approach was reliable for the detection of *Candida*, as 95.1% of *Candida* isolates tested were identified to the species level with 100% specificity. In addition, species-level identification of six medically relevant *Trichosporon* isolates was achieved using a highly variable 12 bp region within the ITS 1 and 2 regions (21). Gaskell *et al* investigated sequence variation in ITS regions to distinguish *Aspergillus* from other allergenic molds (7). They found little variation between *Aspergillus* and *Penicillium* within the ITS 2 region but concluded the ITS 1 region may be sufficient for identification. Although *Penicillium capsulatum* and *P. glabrum* exhibited the highest sequence similarity to *Aspergillus* species in our study, the presence of a 10 bp sequence variation within the ITS 2 region allowed these species to be readily distinguished. We therefore concluded that both the ITS 1 and 2 regions were necessary for species-level identification. A limited number of strains were available for some *Aspergillus* species, particularly *A. ustus*, which was not previously listed in the GenBank database. Although incomplete, the sequence of GenBank sequences of non-referenced strains showed little difference from ATCC referenced strains.

Variation in ITS 2 amplicon size was used by Turenne *et al.* to identify clinically important fungi using capillary electrophoresis (CE) for separation and identification (23). They tested 56 fungi and were able to identify 48 at the species level. Similar to our results, they found only a two nucleotide base difference when comparing the length of *A. flavus*, *A. niger*, and *Fusarium solani* ITS amplicons. This suggested that amplicon length may not be sufficiently different to distinguish species. We also found *A. niger* and *A. terreus* amplicons to be similar in length. The resolution

of CE is approximately two nucleotides for amplicons greater than 250 bases in length. It is not clear whether the technical limitations of CE make it a reliable method for species-level identification of *Aspergillus*.

The comparison of ITS 1 - 5.8S - ITS 2 region sequences between referenced and clinical isolates of six *Aspergillus* species revealed several areas of sequence variation. The inclusion of the 5.8S rRNA gene sequence had minimal impact on the overall comparison since there is little inter-species variation in this region. In our study, the intra-species variation among clinical and pathogenic referenced *Aspergillus* strains was less than 1%. This is consistent with the phylogenetic study by Sugita, et al. of the *Trichosporon* species where less than 1% of nucleotide bases were different among various strains of the same species (21).

Gaskell *et al.* have previously shown that *Alternaria*, *Penicillium*, *Cladosporium*, and *Aspergillus* could be differentiated at the genus level on the basis of ITS sequence analysis (7). The question remained however, whether ITS sequences could be used to identify any fungus that may be recovered clinically, including those that may be environmental contaminants. In our study, a BLAST search of all GenBank sequences was conducted using the six referenced *Aspergillus* species ITS sequences. Sequence similarities of less than 89.6% were seen when comparing the ITS region sequences of *A. fumigatus* to those of other genera, including opportunistic fungi or true pathogenic fungi listed in Table 4. This search also identified 2 species, *A. nidulans* and *A. niger*, that had sequence similarity of 99% with other opportunistic fungi.

A. nidulans (deposited in GenBank as *Emericella nidulans*) ITS sequences had 99% sequence similarity with *Emericella quadrilineata*. However, *E. quadrilineata* has not

been reported as a cause of invasive disease in humans. *A. niger* ITS sequences were found to be similar to non-referenced isolates of *A. phoenicis*, *A. tubigensis*, *Arthrobotrys* species, and *Gliocladium cibotii*. The *A. niger* aggregate includes two subgroups and at least 14 species, including *A. phoenicis* and *A. tubigensis* , that are morphologically indistinguishable. By contrast, *Gliocladium* and *Arthrobotrys* species have morphological features distinct from *A. niger*. Again, none of these species have been associated with invasive disease and their medical importance is unknown (18). Additional studies are in progress to confirm the ITS sequences of referenced isolates of these infrequently encountered fungal species. Overall, the present results showed that ITS sequence analysis can be used to exclude fungal genera which may be considered in the differential diagnosis of a patient with invasive mycosis. However, the sequence similarity of 99% with some genera and species indicated that the BLAST bit score would be needed to identify clinical isolates of *Aspergillus* to the species level. A correct identification of clinical isolates of *A. niger* and *A. nidulans* was made using the highest bit score of listed species from the BLAST search. This demonstrated that ITS 1 and 2 sequence analysis can be used for recognition of many fungal genera, including those that do not typically cause invasive disease such as airborne allergenic fungi.

Our studies showed that it was not necessary to clone the PCR products to obtain an accurate reading of the sequence. The elimination of this step allowed for direct automated sequencing of PCR products and significantly reduced the amount of time involved in obtaining a result. The ability to sample small (approximately 2 mm^2) portions of the culture contributed significantly to rapid identification. Colonies of this size generally cannot be used for morphologic identification and in

most cases the specimen must be incubated for 5 days or longer. The ability to rapidly and accurately identify *Aspergillus* species from blinded samples, with results available within 48 h, confirmed the value of this approach. Several issues may affect the time required to obtain a result, including the availability of a dedicated sequencer. The need to repeat the sequencing procedure due to gel compression or contamination may also delay the process. Although automated sequencing and analysis provided accurate discrimination of *Aspergillus* from other fungi, a probe based DNA hybridization approach has been described for other organisms and may be more cost effective in the future (6, 19).

Identification of medically important *Aspergillus* species from short-term culture using nucleic acid sequence analysis of the ITS 1 and 2 regions in combination with a BLAST bit score, is a reliable and efficient method that provides earlier identification than standard culture methods. The identification of rarely encountered opportunistic organisms following sequence analysis should prompt a review of the sequence data and correlation with clinical findings. Investigations are in progress to determine whether the method has utility for direct identification of fungi in tissue sections where histologic evidence of a fungus exists. Additional studies are needed to demonstrate whether identification of *Aspergillus* at the species level will improve patient outcome through the selection of more effective antifungal therapy.

EXAMPLE 2

INTERNAL TRANSCRIBED SPACER REGION SEQUENCES FOR IDENTIFYING ADDITIONAL CLINICALLY RELEVANT SPECIES OF FUNGUS

As demonstrated in Example 1 use of a universal

primer set to amplify the ITS regions of the fungal rRNA gene followed by sequence analysis of the resulting amplicon facilitates the species specific identification of fungi. Additional sequences have been determined using the described primers for the additional fungi listed in Addendum 1.

ADDENDUM 1

SEQ ID NO: 1
5' primer ITS 1th
GGAAGTAAAAGTCGTAACAAGG

SEQ ID NO: 2
3' primer ITS 4th
GTATCCCTACCTGATCCGAGG

SEQ ID NO: 3
Aspergillus ustus GenBank Accession No: AF157507
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
AGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGTCCGTAGGTGAACCTG
CGGAAGGATCATTACCGAGTGCAGGTCTGCCCCGGGCAGGCCTAACCTCCCACCCGTGAATAC
CTGACCAACGTTGCTTCGGCGGTGCGCCCCTCCGGGGGTAGCCGCCGGAGACCACACCGAACC
TCCTGTCTTTAGTGTTGTCTGAGCTTGATAGCAAACCTATTAAAACTTTCAACAATGGATCTC
TTGGTTCCGGCATCGATGAAGAACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTCC
GAGCGTCATTGCTGCCCTTCAAGCCCGGCTTGTGTGTTGGGTCGTCGTCCCCTCCGGGGGACG
GGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCT
CGATTAGGGCCGGCCGGGCGCCAGCCGGCGTCTCCAACCTTTTATTTTACCAGGTTGACCTCG
GATCAGGTAGGGATAC

SEQ ID NO: 4
Aspergillus terreus Genbank Accession No:AF138290
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
AGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGTCCGTAGGTGAACCTG
CGGAAGGATCATTACCGAGTGCGGGTCTTTATGGCCCAACCTCCCACCCGTGACTATTGTACC
TTGTTGCTTCGGCGGGCCCGCCAGCGTTGCTGGCCGCCGGGGGGCGACTCGCCCCCGGGCCCG
TGCCCGCCGGAGACCCCAACATGAACCCTGTTCTGAAAGCTTGCAGTCTGAGTGTGATTCTTT
GCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA
AATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGC
GCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTG
TGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCG
TCCGGTCCTCGAGCGTATGGGGCTTCGTCTTCCGCTCCGTAGGCCCGGCCGGCGCCCGCCGAC
GCATTTATTTGCAACTTGTTTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATAC

SEQ ID NO: 5
Aspergillus niger GenBank Accession No:AF138904
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
AGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGTCCGTAGGTGAACCTG
CGGAAGGATCATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTGTACC
CTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCC
GTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATG
CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA
ATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCG
CCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGT
GTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTC
CGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGT
TTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATAC

SEQ ID NO: 6
Aspergillus nidulans GenBank Accession No:AF138288
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
AGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGTCCGTAGGTGAACCTG
CGGAAGGATCATTACCGAGTGCGGGCTGCCTCCGGGCGCCCAACCTCCCACCCGTGACTACTA
ACACTGTTGCTTCGGCGGGGAGCCCCCCAGGGGCGAGCCGCCGGGGACCACTGAACTTCATGC
CTGAGAGTGATGCAGTCTGAGCCTGAATACAAATCAGTCAAAACTTTCAACAATGGATCTCTT
GGTTCCGGCATCGATGAAGAACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGA
GCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGTCGTCCCCCCCGGGGGACGGGC
CCGAAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGA
TTAGGGCCGGCCGGGCGCCAGCCGGCGTCTCCAACCTTATTTTTCTCAGGTTGACCTCGGATC
AGGTAGGGATAC

SEQ ID NO: 7
Aspergillus fumigatus GenBank Accession No:AF138288
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
AGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGTCCGTAGGTGAACCTG
CGGAAGGATCATTACCGAGTGAGGGCCCTTTGGGTCCAACCTCCCACCCGTGTCTATCGTACC
TTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCTTGCGCCCCCGGGCCCG
CGCCCGCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCGT
AATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA
TGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGC
CCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTG
TGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCC
GGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGCCGGCGCCAGCCGACACC
CAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATAC

SEQ ID NO: 8
Aspergillus flavus GenBank Accession No:AF138287
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
AGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGTCCGTAGGTGAACCTG
CGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGT
ACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGC

GCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAAT
CAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGC
GATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCC
CTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTG
TTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGA
TCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAA
ATCAATCTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATAC

SEQ ID NO: 9
Pseudallescheria boydii GenBank Accession No: AF181558
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
AGGGCCAGCCATACGGACGGCGCTACTCGCGTACAACGTCTCTGGCGTCCGTAGGTGAACCTG
CGGAGGGATCATTACAGAGTTACTACTCCAAACCCATTGTGGGAAGTAAAAGTCGTAACAAGG
TTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCAAGGGCCAGCCATACGGACGGCG
CTACTCGCGTACAACGTCTCTGGCGAACCTTACCTATGTTCTGTTGCCTCGGCGGCGTGGTCA
GCGCCCCCTCTGAAAAGAGGACGATGTCCTCCCGCCGGCAGCACCAAACTCTTTGAATTTTAC
AGCGGATCACAGTTCTGATTTGAAAACAAAAAACAAGTTAAAACTTTCAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCGGCAGTAATCTGCCGGGCATGCCTGTCCGA
GCGTCATTTCAACCCTCGAACCTCCGTTTCCTCAGGGAAGCTCAGGGTCGGTGTTGGGGCGCT
ACGGCGAGTCTTCGCGACCCTCCGTAGGCCCTGAAATACAGTGGCGGTCCCGCCGCGGTTGCC
TTCTGCGTAGTAAGTCTCTTTTGCAAGCTCGCATTGGGTCCCGGCGGAGGCCTGCCGTCAAAC
CACCTATAACTCCAGATGGTTGACCTCGGATCAGGTAGGGTAC

SEQ ID NO: 10
Fusarium solani GenBank Accession No:AF165874
(deposited as Nectria haemotococca)
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTCGAGTTATAC
AACTCATCAACCCTGTGAACATACCTATAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAAC
ACGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCTATAATGTTTCTTCTGAGTAAACA
AGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGC
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SEQ ID NO: 11
Fusarium oxysporum GenBank Accession No: AF165875
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GGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGC
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SEQ ID NO: 12
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SEQ ID NO: 13
Malassezia furfur GenBank Accession No:AF246896
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
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C

SEQ ID NO: 14
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SEQ ID NO: 15
Cladophialophora bantiana GenBank Accession No:AF131079
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SEQ ID NO: 16
Gymnascella hyalinaspora GenBank Accession No:AF129854
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SEQ ID NO: 17
Blastomyces dermatitides GenBank Accession No: AF183912
(deposited as *Ajellomyces dermatitidis*)
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SEQ ID NO: 18
Histoplasma duboisii GenBank Accession No: AF162917
(deposited *Ajellomyces capsulatus*)
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
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SEQ ID NO: 19
Histoplasma capsulatum GenBank Accession No:AF156892
(deposited as *Ajellomyces capsulatus*)
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GTCCGCCGGGGACACCGCAAGAACCGTCGGTGAACGATTGGCGTCTGAGCATAAGAGCGATAA
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SEQ ID NO: 20
Cryptococcus neoformans GenBank Accession No:AF162916
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AGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCAT
CTCTTTTTGT

SEQ ID NO: 21
Issatchenkia orientalis GenBank Accession No: AF246989
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GACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGCGAACTAGACTTTTTTTCAGGGACGCTTGGC
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SEQ ID NO: 22
Candida albicans GenBank Accession No: AF217609
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CACCACGTATATCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCAT
ATCAATAAGCGGAGGA

SEQ ID NO: 23
Candida lusitaniae GenBank Accession No: AF172262
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ACTACCCG

SEQ ID NO: 24
Candida glabrata GenBank Accession No: AF167993
AAGAATTTAATTGATTTGTCTGAGCTCGGAGAGAGACATCTCTGGGGAGGACCAGTGTGACAC
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SEQ ID NO:25
Penicillium spp.
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AAGGCAGCGGCGGCACCGCCTTCCCGGTCCTCCGAGCCTTATGGGGCTTTGTTCACCCCGCTC
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ATCANGTTAGGGATAC

SEQ ID NO: 26
Malbranchia spp.
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
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GCGCCCGCCAGAGATACACTGAACCCTTTGTGAAATTGGACGTCTGAGTTGATGATCAATCAT
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TATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCAACCCTCAAGCGCGGCTTGTGTGTTGGG
CCTCGTCCCCCGTGGACGTGCCCGAAAGGCAGTGGCGGCGTCCGTTTCGGTGCCCGAGCGTAT
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GACCTCGGATCAGGTAGGGATACC

SEQ ID NO: 27
Arthrogrothilus spp.
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
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GTAGGTCTGGATGACTCTCGTGCTCTCTGAGTGCGGATGCGAGGATTGCCCTCTTGAGGTGTC
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AGCTCCGTCTATGGTNGTGATAAATTATCTACGCCCGTTGGACNGTTTTAAAACTCCCTTCTA
ACCGTCCCGCAANGANAATANCTTTT

SEQ ID NO: 28
Cylindrocarpon destructans
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
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TTCTATTCTTCTTAGGTTGACCTCGGATCAGGTAGGGATACC

SEQ ID NO: 29
Sporothrix schenkii
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CGAGTGGCGGGCCCTGTGGAAGGCTCCGAGCGCAGTACCGAACGCATGTTCTCCCCTCGCTCC
GGACGCCCCCCAGGCGCCCTGCCGTGAAAACGCGCATGACGCGCAGCTCTTTTTACAAGGTTG
ACCTCGCCGCTGACCTCGGATCAGTAGGGAATAC

SEQ ID NO: 30
Penicillium marnefeii
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
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AACTTTATTTTTCTAAGGTTGACCTTGGATCAGGTAGGGATACCCGCTGCCTCGGATCAGGTA
GGAATAC

SEQ ID NO: 31
Coccidiodes immitis
GGAAGTAAAAGTCGTAACAAGGTTTCTGTAGGTGAACCTGCAGAAGGATCATTAGTGAAAGCA
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GCTGCGCACCTCCCCCGCGGGGGTTCGCGCGGTCCGTACCTCCCACCCGTGTTTACTGAACCA
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ATAGCAAAAATCAAACAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACG
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CATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTGCAAACCCTTCAAGC
ACGGCTTGTGTGTTGGGCCAACGTCCCCGCTTGTGTGGACGGGCCTGAAATGCAGTGGCGGCA
CCGAGTTCCTGGTGTCTGAGTGTATGGGAAATCACTTCATCGCTCAAAGACCCGATCGGGGCC
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CCTCGGATCAGGTAGGAATAC

SEQ ID NO: 32
Candida tropicalis
GGAAGTAAAAAGTCGTAACAAGGTTTCCGAGGNGAACCTGCGGAAGGATCNTTACTGATTTGC
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CCANAGGTTANAACTAAACCNAACTTTTTNTTTACAGTCNAACTTNATTTATTATTACNANAG
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GTATTCCAAANGGCANGCCTGTTTNANCGTCATTTNTCCCNCNAACCCCCGGGNTTGGTGTTN
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TATCCNAAAACGCTTATTTTGCTAGGGCCACCACAATTTATTTCAAACTTGACCCA

SEQ ID NO: 33
Candida parapsilosis
GGAAGTAAAAAGTCGGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGAATG
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GGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTTGAAAGAAAGGCGGAGTATAAACTAATGGA
TAGGTTTTTTCCACTCATTGGTACAAACTCCAAAACTTCTTCCAAATTCGACCCA

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While preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.